

AMENDMENTS TO THE SPECIFICATION

IN THE SPECIFICATION

On page 2, line 31, please replace the original paragraph with the following amended paragraph:

--We have now designed improved methods for peptide production and modification by exploiting the intein-mediated protein splicing as a method to rapidly produce phage display peptides in a soluble form. The methods of the invention are particularly useful in producing peptides having disulfide bridges, the peptide cleavage being carried out using temperature/pH-inducible intein splicing. As an example the production of the dodecapeptide inhibitor of gelatinases CTTHWGFTLC (CTT) (SEQ ID NO: 10) is described. CTT is a disulfide bond-containing low molecular weight peptide that has been discovered by screening random peptide libraries displayed on filamentous phage. --

On page 3, line 20, please replace the original paragraph with the following amended paragraph:

-- We thus used intein-mediated protein cleavage reaction for the generation of recombinant peptides in *E. coli*. The method allowed rapid production and purification of the ten-residue long gelatinase inhibitor peptide CTTHWGFTLC (SEQ ID NO: 10) in milligram quantities. Alanine scanning mutagenesis of the peptide showed that the tryptophan residue is central for the gelatinase inhibitory activity. Intein cleavage also occurred after biosynthetic incorporation of hydroxylated and fluorinated tryptophan analogues into the intein fusion protein. The analogues were incorporated efficiently using a protein expression strain converted to a tryptophan auxotroph by insertional mutagenesis using *in vitro* assembled bacteriophage Mu DNA transposition complexes. All tryptophan analogue-containing peptides retained the gelatinase inhibitory activity. 5-

fluorotryptophan-containing peptide showed enhanced stability in serum and was more potent inhibitor of tumor cell invasion than the wild type CTTHWGFTLC (SEQ ID NO: 10) peptide. These studies open new possibilities to modify peptides and improve their activity by biosynthetic incorporation of unnatural amino acids. Collectively these studies show that intein-mediated expression of peptides is a versatile tool for peptide design and may enable development of highly active peptides with potential therapeutic applications.--

On page 4, line 4, please replace the original paragraph with the following amended paragraph:

-- Furthermore, we performed phage selection using proMMP-9 as a target. After three rounds of selection, we cloned the resulting peptides in an intein vector using a pair of oligonucleotide primers that were designed so that any phage peptide insert can be amplified without the knowledge of the peptide sequence. The resulting peptides have a sequence ADGA-(X)n-GAAG (SEQ ID NO: 25), where the ADGA (SEQ ID NO: 8) and GAAG (SEQ ID NO: 9) amino acid sequences are derived from the phage and (X)n is the peptide insertion. As an example, two such peptides were successfully expressed and their specificity could be shown by inhibition of phage binding.--

On page 5, line 21, please replace the original paragraph with the following amended paragraph:

-- We thus designed the following universal primers.

(1) Intein Fwd SapI primer having the sequence: CCT TTC TGC TCT TCC AAC GCC GAC GGG GCT (SEQ ID NO: 1). This primer will add amino acids ADGA (SEQ ID NO: 8) from the phage to the peptide.

(2) Intein Rev *PstI* primer having the sequence: ACT TTC AAC CTG CAG TTA CCC AGC GGC CCC (SEQ ID NO: 2). This primer will add amino acids GAAG (SEQ ID NO: 9) from the phage to the peptide. --

On page 6, line 27, please replace the original paragraph with the following amended paragraph:

-- Abbreviations:

CTT: CTTHWGFTLC (SEQ ID NO: 10) peptide (Koivunen *et al.*, 1999a)

iCTT: recombinant CTTHWGFTLC (SEQ ID NO: 10) peptide;

STT: STTHWGFTLS (SEQ ID NO: 11) peptide;

MMP: matrix metalloproteinase;

5OH-Trp: 5-hydroxytryptophan;

5F-Trp: 5-fluorotryptophan;

6F-Trp: 6-fluorotryptophan;

7A-Trp: 7-azatryptophan.

On page 9, line 27, please replace the original paragraph with the following amended paragraph:

--Cloning of the intein-peptide fusions. A synthetic oligonucleotide 5'-GGTGGTGCTCTTCCAACTGTACGACCCATTGGGGATTACTTATGTTAACTGCAGGCG-3' (SEQ ID NO: 3) encoding the CTTHWGFTLC (SEQ ID NO: 10) peptide was converted to double stranded form using Dynazyme II DNA polymerase (Finnzymes, Espoo, Finland) with a primer 5'-CGCCTGCAGTTAAC-3' (SEQ ID NO: 4), and digested with *SapI* and *PstI*. Purified insert was ligated in frame to *SapI*-*PstI*-digested pTwin vector backbone (New England Biolabs) (Evans *et al.*, 1999). The presence of the correct insert was verified by sequence analysis. Similar cloning strategy

was used to prepare the alanine-mutant peptides using codon GCG for alanine. For the cloning of any phage peptide inserts, universal oligonucleotides 5'-CCT TTC TGC TCT TCC AAC GCC GAC GGG GCT-3' (SEQ ID NO: 1) (Intein Fwd *SapI*), 5'-ACT TTC AAC CTG CAG TTA CCC AGC GGC CCC-3' (SEQ ID NO: 2) (Intein Rev *PstI*) were used. For the hydrophilic CTT peptide library, a synthetic degenerate oligonucleotide 5'-GGTGGTTGCTCTTCCAACGGCCGCCVAVVAVTATVAV GGCTGTACCACCCATTACTTATGTTAACTGCAGGCG-3' (where V is A, C or G) (SEQ ID NO: 5) was prepared, and converted to double-stranded DNA with the same primer as the normal CTT peptide.--

On page 10, line 28, please replace the original paragraph with the following amended paragraph:

-- **Generation of tryptophan auxotrophic *E. coli* ER2566 mutant.** *In vitro* assembled bacteriophage Mu DNA transposition complexes were prepared essentially as described previously (Lamberg *et al.*, 2002). Briefly, 1.1 pmol transposon DNA containing a kanamycin resistance gene and 4.9 pmol MuA protein were mixed in 20 μ l of 150 mM Tris-HCl (pH 6.0) / 50% glycerol / 0.025% Triton X-100 / 150 mM NaCl / 0.1mM EDTA. The transposition complex assembly reaction was carried out at 30°C for 2 h. The complexes were electroporated as 1:8 or 1:16 dilutions into electrocompetent *E. coli* ER2566 and plated on LB plates containing 50 μ g/ml kanamycin. The clones obtained were replica-plated on M9 minimal plates and M9 plates containing 1 mM DL-tryptophan (Sigma). A clone named ER2566/Trp82 requiring Trp for growth was chosen for further studies. To determine the transposon insertion site, the chromosomal DNA was isolated with genomic DNA isolation kit (Qiagen) and digested with *PstI*. The resulting genomic fragments were ligated with *PstI* digested pUC19 plasmid and transformants selected in the presence of kanamycin. The DNA sequences of transposon borders were determined by sequencing with transposon specific primers 5'-ATCAGCGGCCGCGATCC-3' (SEQ ID NO: 6) and 5'-TTATTCGGTCGA AAAGGATCC-3' (SEQ ID NO: 7). The genomic location of the insertion was identified using the BLAST search. --

On page 12, line 4, please replace the original paragraph with the following amended paragraph:

-- **Gelatinase inhibition assays.** Gelatinases proMMP-2 and proMMP-9 (Roche) were activated with p-aminophenylmercuric acetate or trypsin, respectively, and then incubated in the presence or absence of each peptide to be tested for 30 min. The gelatinase inhibitory activity was determined using the following three assays: (i) The degradation of biotinylated gelatin was examined using a gelatinase activity kit according to the manufacturer's instructions (Roche). (ii) The degradation of a MMP-2 specific fluorescent peptide substrate MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂ (SEQ ID NO: 26) (Calbiochem) (2.5μM final concentration) was followed using MOS-250 spectrofluorometer (Bio-Logic SA, Claix, France) with 330 nm excitation and 390 nm emission. (iii) The degradation of β-casein was studied by incubating activated MMP-2 with 0.1 mg/ml concentration of β-casein for 2 h at 37°C, after which the samples were analyzed on a 15% SDS-PAGE gel.--

On page 15, line 9, please replace the original paragraph with the following amended paragraph:

-- **Table 1. Recombinant peptides prepared by the intein system.**

Peptide	Sequence	MASS, DA	
		Calc. *	Obs.
ICTT	CTTHWGFTLC (<u>SEQ ID NO: 10</u>)	1166.4	1166.5
T1→A	CATHWGFTLC (<u>SEQ ID NO: 12</u>)	1136.3	1136.4
T2→A	CTAHWGFTLC (<u>SEQ ID NO: 13</u>)	1136.3	1136.4
H→A	CTTAWGFTLC (<u>SEQ ID NO: 14</u>)	1100.3	1100.5
W→A	CTTHAGFTLC (<u>SEQ ID NO: 15</u>)	1051.2	1051.4
G→A	CTTHWAFTLC (<u>SEQ ID NO: 16</u>)	1180.4	1180.5
F→A	CTTHWGATLC (<u>SEQ ID NO: 17</u>)	1090.3	1090.4
T3→A	CTTHWGFLAC (<u>SEQ ID NO: 19</u>)	1136.3	1136.5
L→A	CTTHWGFTAC (<u>SEQ ID NO: 27</u>)	1124.3	1124.4
5OH-CTT	CTTH(5OHW)GFTLC (<u>SEQ ID NO: 20</u>)	1182.4	1182.4

5F-CTT	CTTH(5FW)GFTLC (<u>SEQ ID NO: 20</u>)	1184.4	1184.3
6F- CTT	CTTH(6FW)GFTLC (<u>SEQ ID NO: 20</u>)	1184.4	1184.2

On page 15, line 14, please replace the original paragraph with the following amended paragraph:

--Functional analysis of the gelatinase inhibitory peptides

The gelatinase inhibitory activity of iCTT was found to be identical with that of the chemically synthesized CTT in several assays. In gelatin degradation assay, iCTT and CTT exhibited a similar dose dependency, the IC50 values being 20 μ M for both MMP-2 and MMP-9 inhibition (Fig. 1A and data not shown). The non-cyclic synthetic control peptide STTHWGFTLS (STT) (SEQ ID NO: 11) was several fold less active than iCTT. --

On page 17, line 18, please replace the original paragraph with the following amended paragraph:

-- The gelatinase inhibitory activities of the modified CTT peptides were tested in the β -casein degradation assay (Fig. 3A) and the gelatin degradation assay (data not shown). No significant differences in the gelatinase inhibitory activity were seen in these assays. At a 100 μ M concentration 5OH-Trp, 5F-Trp and 6F-Trp containing peptides inhibited MMP-2 with a similar efficiency as iCTT does with nearly complete inhibition of casein degradation. As amino acid analogues can contribute to the protease sensitivity of the peptides, we next studied the stability of the peptides by incubating them in normal human serum. To determine the peptide levels, we used anti-CTT antibody that also recognized the 5F-Trp containing peptide. The 5F-Trp containing peptide was more stable in serum

with a half-life of 3 hours in comparison to the 0.5 hour half-life of the wild type CTT peptide (Fig. 3B). We could not determine the half-lives of 5OH-Trp and 6F-Trp containing peptides as the anti-CTT antibody recognized these peptides only weakly in the presence of serum. The CTT antibody was highly specific as cyclic control peptides CERGGLETSC (SEQ ID NO: 21) and CPCFLLGCC (SEQ ID NO: 22) did not react with the anti-CTT antibody. No difference between the stability of CTT and 5F-Trp containing peptide was seen in cell culture medium supplemented with 10% heat-inactivated fetal calf serum (data not shown).--

On page 18, line 12, please replace the original paragraph with the following amended paragraph:

-- To achieve labeling of the CTT peptide with radioactive iodine, peptides with additional tyrosine were prepared by chemical synthesis. However, these peptides were found insoluble in water, although the CTT peptide itself is water-soluble. Thus, we prepared a peptide library with intein system to screen for a water-soluble tyrosine-containing CTT peptide. A degenerate oligonucleotide having randomized amino acids coding for polar amino acids were used to potentially enhance the solubility of the tyrosine containing CTT peptide. The resulting library coded for peptides GRXXYXGCTTHWGFTLC (SEQ ID NO: 23), wherein X is any hydrophilic amino acid. First an oligonucleotide 5'-GGTGGTTGCTCTTCCAACGGCCGCCVAVVAVTATVAVGGCTGTACCA CCCATTACTTATGTTAACTGCAGGCG-3' (SEQ ID NO: 5) was designed, and prepared by combinatorial synthesis using an oligonucleotide synthesizer. The oligonucleotide contained three VAV codons, (wherein V is G or A or C), which code for hydrophilic amino acids.--

On page 18, line 25, please replace the original paragraph with the following amended paragraph:

-- The oligonucleotide was made double-stranded using PCR. The PCR product was digested with PstI and SapI and cloned to TWIN2 intein vector (New England Biolabs), digested also with PstI and SapI. This DNA construct was electroporated into MC1061 competent cells. The library obtained contained 216 variations of the CTT-peptide. The plasmid vectors were extracted from the pool of at least 216 independent clones of MC1061. The plasmids were then electroporated to ER2566 cells enabling the production of inteins. The cells harboring the plasmids were plated on LB plates containing ampicillin. 10 independent clones were pooled to one single pool. These 10 clones in one pool were cultured and the peptides were expressed and purified with chitin affinity column and reverse-phase C18 column. Each pool of peptides was tested for activity. The solubility in PBS was also tested. Two clones of two pools with distinct activity and good solubility were cultured, and the peptides were purified as described above. One of these peptides (having the sequence GRENYHGCTTHWGFTLC (SEQ ID NO: 24)) was more soluble into water or PBS than the original peptide, and it was active (Figure 5). The plasmid coding for this peptide was sequenced and the peptide was synthesized with chemical synthesis.--

On page 19, line 31, please replace the original paragraph with the following amended paragraph:

-- The commercially available tryptophan analogues 5-hydroxy-L-tryptophan (5OH), 5-fluoro-DL-tryptophan (5FW), 6-fluoro-DL-tryptophan (6FW) and DL-7-azatryptophan (7AW) were first tested for the incorporation efficiency into phage particles. Fig. (8). The tryptophan auxotrophic MB5F strain was infected with fUSE5 phage carrying the CTTHWGFTLC (SEQ ID NO: 10) peptide. The

infected bacteria were first cultured in a defined medium and then shifted to a medium containing the amino acid analogue to be tested. As a control, the bacteria were transferred to a medium lacking tryptophan or the analogues. After an overnight culture, the titer of the CTT-fUSE5 phage in the culture supernatant was measured by infecting the *E. coli* K91/kan. Roughly equal number of infective particles was obtained with 5FW and 6FW as compared to tryptophan. The combination of 5FW and 6FW also yielded phage particles efficiently. In contrast, the 5OH and 7AW supported the phage production poorly. Fig. (9A).--